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STUDIES ON THE IMMUNOCHEMICAL TECHNIQUES FOR DETECTION OF SELECTED FUNGAL AND DINOFLAGELLATE TOXINS

Annual Progress Report
(November 1, 1981-July 31, 1982)

F. S. Chu

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Food Research Institute and Department
of Food Microbiology and Toxicology
University of Wisconsin
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Foreword

The following is the first annual report (9 months) of the work performed under contract No. DAMD17-82-C-2021 during the period of Nov. 1, 1981 to July 31, 1982. The work was carried out at the Food Research Institute of the University of Wisconsin-Madison under the direction of the principal investigator, Dr. F. S. Chu and co-principal investigator, Dr. E. J. Schantz. The contract officer is Dr. Robert Wannemacher.

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ABSTRACT

During the first nine months, effective methods for the preparation of hemisuccinate (HS) and hemiglutarate (HG) of T-2 toxin, HS of diacetoxyscirpenol (DAS), and O-carboxymethyl oxime (O-CM) of vomitoxin (deoxynivalenol, VT) were developed. Methods for the preparation of reduced saxitoxin (STX) as well as HS of reduced saxitoxin (STXOL) were also established. These derivatives were chemically characterized and were conjugated to bovine serum albumin (BSA) for subsequent immunization. They have also been conjugated to horseradish peroxidase (HRP) for direct enzyme linked immunosorbent assay (ELISA) and to polylysine and hemocyanin for indirect ELISA. The toxicity of T-2 HS (LD_{50}) was determined in mice, and was found to be around 7.5 mg/kg as compared with T-2 toxin which had a LD_{50} around 3-4 mg/kg. For antibody production, 26 rabbits were immunized with various toxin-BSA conjugates. The antibody titers were measured by either a RIA method using tritiated reduced VT or ^{3}H-T-2 toxin or tritiated STXOL, or by both direct and indirect ELISA methods using the toxin-HRP or goat antirabbits IgG-HRP as the enzyme marker. Antibody titers varied considerably with the conjugates used but none was found to be very immunogenic. Whereas antibody titers against STX and VT were demonstrated by an indirect ELISA method for most immunized rabbits, the titers were very weak as determined by the direct ELISA. During the present contracting period, a total 143 mg of T-2 BSA conjugate with varied degrees of T-2 toxin per mole of BSA, 5 mg T-2 enzymes, 4 mg of T-2 polylysine, 34 ml of antisera against T-2 toxin, 20 mg of T 2-hemocynin, and 1 mCi of ^{3}H-T-2 toxin were prepared and delivered.

I. INTRODUCTION

The goal of this contract is to develop a method for the production of antibodies against several selected mycotoxins and dinoflagellate phytotoxins and subsequently to develop a radioimmunoassay (RIA) or an enzyme-linked immunosorbent assay (ELISA) for toxin determination. To achieve the main objective, the following specific tasks were planned:

(a) development of methods for conjugation of STX and its related dinoflagellate toxins to protein carrier;

(b) development of methods for conjugation of VT and other related trichothecene mycotoxins to protein carrier;

(c) produce antibodies against the toxin-protein conjugates;

(d) development of RIA for mycotoxins and their application for analysis of these toxins in military foods and body fluids of military personnel.

(e) investigation of the in vitro and in vivo neutralization of mycotoxins and STX by antibody;

(f) deliver hapten, antibody, and enzyme-linked toxin to the U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID).

In the first nine months, our work has been directed to four major areas with emphasis on four major toxins including T-2, VT, STX and DAS:

(a) preparation of enough immunochemical reagents (T-2 toxin related) for USAMRIID in-house research;

(b) studies on the optimal conditions for the preparation of the toxin derivatives as well as effective method for conjugation to proteins and enzymes;

(c) studies on the optimal condition for the production of antibodies against these toxins;

(d) development of RIA and ELISA for the analysis of these toxins.

The progress of the first nine months of work is as follows.

II. ASSESSMENT OF WORK DONE

A. Studies on the preparation toxins and their protein conjugate:

(a) An efficient method for the preparation and purification of VT and O-carboxymethyl VT (O-CM-VT) was developed. Both preparations were chemically characterized. The purified O-CM-VT was conjugated to BSA and subsequently used in the immunization. O-carboxymethyl oxime of VT was also conjugated to horseradish peroxidase (HRP) and to polylysine for ELISA of antibody titers.

In addition, a reduced tritiated VT was prepared and was used in the RIA of VT antibody titers.

(b) For T-2 toxin, a T-2 hemisuccinate (T-2 HS) and a T-2 hemiglutarate (T-2 HG) were prepared and chemically characterized. In addition to conjugate of T-2 HS to BSA under various conditions to give conjugates with different molar ratio of T-2/BSA, T-2 HS was also conjugated to hemocyanin, polylysine and to HRP for direct and indirect ELISAs. T-2 HG was conjugated to BSA which was then used for immunization.

(c) An efficient procedure for the preparation of reduced STX (STXOL) and STXOL HS was established. STXOL-HS was then conjugated to either BSA for immunization or to HRP for ELISA studies. A radioactive STX was prepared by reduction of STX with tritiated $NaBH_4$. Efforts for the preparation of O-CM-ST were made.

B. Production of antibodies against selected mycotoxins and STX:

During the past 9 months, 26 rabbits were immunized with different toxin-protein conjugates. The following conjugates were used:

(a) T-2 HS EDA BSA (2 rabbits)

(b) T-2 HS BSA (3 rabbits)

(c) T-2 HG BSA (3 rabbits)

(d) O-CM-VT-EDA-BSA (mixed anhydride method; 6 rabbits)

(e) O-CM-VT-EDA-BSA (water soluble carbodiimide method; 3 rabbits)

(f) O-CM-VT-BSA (water soluble carbodiimide; 3 rabbits)

(g) DAS-HS BSA (3 rabbits)

(h) STX-HS BSA (3 rabbits)

C. Development of indirect immunoassay for mycotoxin and STX:

A new sensitive indirect enzyme-linked immunoassay for determination of antibody titers against mycotoxin and STX and for mycotoxin determination is developed. This technique is very sensitive for antibody determination and requires less antibody in the ELISA of mycotoxin.

D. Preparation of compounds for USAMRIID:

One hundred and forty-three mg of BSA conjugate with varied degree of T-2 toxin per mole of BSA, 5 mg T-2 enzymes, 4 mg of T-2 polylysine, 34 ml of antisera against T-2 toxin, 20 mg of T-hemocynin, and 1 mCi of H3-T-2 toxin were prepared and delivered. Methods for preparation of such compounds are summaried in Table 1.

III. EXPERIMENTAL

A. Preparation of toxin derivatives:

(a) Preparation of T-2 HS, T-2 HG, and DAS HS: The HS and HG of T-2 and DAS were prepared by refluxing respective toxins with succinic anhydride or glutaric anhydride in the presence of 4-dimethylaminopyridine. Tritiated T-2 with high specific radioactivity was prepared by reducing excess amount of 3-dehydro-T-2 toxin with $NaBH_4$ of high specific radioactivity. Separation of the unreacted toxin was achieved by a semipreparative HPLC previously described in our laboratory.

(b) Preparation and characterization of O-CM-VT: Vomitoxin was produced by Fusarium graminearum in rice culture and purified by silica gel chromatography. Three methods were tested for the preparation of O-CM-VT. It was found that a vigrous condition which involved the refluxing of the toxin with carboxymethoxylamine HCl in strong base (5% NaOH) is necessary to give a more complete reaction. Details for the preparation and characterization of O-CM-VT were described in our first quarterly report. O-CM-VT was further purified by preparative TLC and then crystallized in chloroform. Mass spectral analysis revealed that this new compound had a molecular ion of 3694 which is consistent with the molecular weight of O-CM-VT.

(c) Preparation of reduced 8-OH-VT: Reduction of VT was achieved by reduction of VT-with $NaBH_4$ at OC in isopropanol. Thin layer chromatography analysis (silica gel G-60) revealed that 8-OH-VT had a R_f value of 0.61 (ethylacetate:methanol:acetic acid;v/v/v, 8:2:0.2) as compared with a R_f of 0.72 for VT. Mass spectral analysis revealed that the reduced product had a molecular ion of 298 confirming that the reduction had occurred. When the reduction was carried out at the room temperature, a product which had a molecular ion of 300 was also observed. This substance which can not be separated from the 8-OH-VT in TLC, probably resulted from further reduction of 8-OH-VT at the 9, 10 position in the VT molecule. The same procedure (OC) was used in the preparation of radiolabeled 8-OH-VT except with excess VT. Unreacted VT was removed from the reaction mixture by a semipreparative HPLC column. Details for the reduction conditions and procedures for purification were given in our 2nd quarter report. The TLC properties of different new trichothecene mycotoxin derivatives are summarized in Table II.

(d) Preparation of STXOL: STXOL was prepared by reduction of STX with $NaBH_4$ using the procedures similar to those described by Koehn et al (Bioorgan. Chem. 10:412, 1981) except that the reaction was carried out at a lower temperature (-5C) and in ethanolic solution. An Amberlite CG-50 column (6 X 30 mm) was used to remove the inorganic salts. After loading the sample, the column was washed with distilled water; STXOL was recovered from the column by eluting with 0.25 N of acetic acid. Reduction products obtained at room temperature were not as clean as those obtained at lower temperatures. Further characterization of STXOL was made by TLC using either C-18 reversed phase TLC plate or Silica gel G-60 plate. STXOL gave a positive reaction with the Weber's reagent at approximately 4 ug per spot but did not show any florescence after spraying with H_2O_2. The Rf values of different STX derivatives in TLC are given in Table III. Tritiated $NaBH_4$ was used in the preparation of radiolabeled STXOL. After reaction, either Amberlite CG-50 or Bio Rex 70 (H^+ form) was used to remove the excess salts.

(e) Preparation of STXOL-HS: Both acid and base catalyzed acylation were tested for the succinylation of STXOL. Since STX is more stable in the acidic condition, we have selected the acid catalyzed reaction. Detailed conditions were presented in our 2nd quarter report. Final removal of the excess succinic acid and succinic anhydride was made by passing the residues through an Amberlite CG-50 column, washing with distilled water and eluting the STX-HS from the column with 0.5N acetic acid. To confirm the acylation reaction, either tritiated-STXOL or ^{14}C succinic anhydride were used. Radioactivity found in the product had the same R_f as the STX-HS.

(f) Preparation of O-CM-STX: Attempts to make the O-CM-STX were made using the same procedures which we have used for the preparation of O-CM of aflatoxin B_1. Three tests were made and the details were given in our 2nd quarterly report. Three new spots, which all had R_f values greater than STX, were found after the STX was refluxed with carboxymethoxyl amine HCL for 24 hours. However, attempts to make the methylated derivative of the suspected O-CM-STX were unsuccessful. In the third quarter, three additional experiments were carried out. Saxitoxin was reacted with carboxymethoxy amine HCl under the following conditions: (i) in the presence of anhydrous sodium acetate with ethanol as solvent; (ii) in the presence of anhydrous sodium acetate and acetic acid with ethanol and benzene as solvent; and (iii) in the presence of triethylamine with ethanol as solvent. However, none of those gave a satisfactory result.

B. Preparation of protein conjugates of T-2 HS, T-2 HG, O-CM-VT, DAS-HS, and STX-HS:

Two approaches were used for conjugation of various toxin derivatives to proteins. In general, mixed anhydride method was used for the conjugation of those derivative to BSA or to a modified BSA, i.e., ethylene diamine modified BSA (EDA-BSA), to yield higher degree of molar ratio of toxin to BSA. Those conjugates were subsequently used for immunization. Water soluble carbodiimide method was used primarily for coupling the toxin derivatives to enzymes. The amount of T-2 toxin to BSA was estimated by determining the residual amino groups in the protein according to the method of Habeeb (Analy. Biochem. 14:328, 1966). Protocols for such preparation were detailed in our 1st and 2nd quarterly reports.

C. Production of antibodies against VT, DAS, T-2 toxin, and STX:

Production of antibodies against various toxins was made by immunizing rabbits with different toxin-BSA conjugates using a multiple injection technique in the initial injection as previously described. At least three rabbits were used for each conjugate. Each rabbit received 300-750 µg of the conjugate in Fruend's complete adjuvant in the initial injections. Booster injections were made at an interval of every 4-6 weeks. The antibody titers were measured by a RIA methods using tritiated reduced toxin derivatives as the radioactive ligand, and also by either a competitive direct ELISA technique using toxin-peroxidase conjugate as the enzyme marker or an indirect ELISA. Examples for antibody titer determination are shown in figures 1 and 2. Details for the antibody production are summarized in Table IV.

D. Development of an indirect ELISA for monitoring the antibody titer against mycotoxin and for mycotoxin analysis.

An indirect enzyme linked immunoassay was developed during the third quarter of the present contract. The assay is based on the principal of binding of IgG for a specific mycotoxin obtained from rabbits with a corresponding mycotoxin-polylysine conjugate which is coated to a solid phase such as the microELISA plate. The antibody bound to the mycotoxin-polylysine solid phase is then reacted with a second antibody, i.e. goat antirabbits IgG, to which an enzyme such HRP was conjugated. The amount of goat antirabbit IgG-peroxidase bound to the rabbit IgG was subsequently determined by reacting with a chromogenic substrate. A typical example for the determination of antibody titer is shown in figure 2. For mycotoxin determination, the free toxin standard or sample to be analyzed is incubated together with the rabbit IgG before incubation with the second antibody-enzyme conjugate. A typical example of displacement curve for standard T-2 toxin is shown in figure 3.

Although the indirect ELISA is very sensitive for monitoring a small amount of antibody present and also requires less antibody for analysis of mycotoxins, there are still some problems associated with this technique. For example, during the second quarter we have prepared a small amount of hemocyanin-T-2 hemisuccinate (HC-T2HS) conjugate (please see details in our second quarterly report) which has been shown to be useful in monitoring of antibody titers for T-2 toxin. However, when we used HC-T2 HS in the indirect ELISA of T-2 toxin, we found that a large amount of T-2 toxin was necessary for the displacement. This problem was later overcome by coating T-2 polylysine to the plate. During the third quarter, we have tested this method very extensively for vomitoxin. Again, we found that a large amount of VT (approximately 100 ng/well) was necessary for displacement.

E. Toxicity of T-2 HS:

The toxicity of T-2 HS were determined during the third quarter. Thirty two mice were divided into 8 groups and each was injected with a varied amount of T-2 or T-2 HS in 0.1 ml of DMSO intraperitoneally. The time of death was recorded. The results as shown in Table V indicate that T-2 HS is less toxic than T-2 toxin. The LD_{50} for T-2 toxin were estimated to be around 7.5 mg/kg and 3-4 mg/kg, respectively.

IV. DISCUSSION

Two scopes of work are covered in this contract. One of which is to prepare enough immunochemical reagents for T-2 toxin according to the methods previously developed in our and other laboratories. The other is to develop new immunochemical methods for other related mycotoxins and saxitoxin. Extensive progress has been made during the contracting period on both areas. Regarding the first scope, we have prepared most required reagents and have worked very closely with the contracting officer. Regarding the second scope, we have developed methods for the preparation of several new toxin derivative and toxin conjugates. To test the feasibility of using those conjugate for antibody production, an extensive immunization program was started. During the investigation, we have also found several problems which are warranted for further studies.

To develop an effective immunoassay or to carry out other immunochemical investigations, high quality and large quantities of specific antiserum is necessary. One of the most critical problems we found is that most of the mycotoxin-conjugates in the trichothecene group are not very immunogenic. For example, whereas we have successfully prepared T-2 BSA conjugate which had a high T-2 to BSA molar ratio (19 moles/mole), studies carried out both in our and in USAMRIID laboratories revealed that the antibody titers were low. In addition, a prolonged immunization schedule is necessary. Similar problems appear also associated with VT and DAS. Since T-2 toxin is an immunosuppression agent and since T-2 HS is also toxic, we rationalized that conjugate with high ratio of T-2 toxin per mole of BSA may not necessarily be adequate for immunization. To overcome this problem, a systematic study for antibody production is necessary. Studies such as immunizing rabbits with conjugates containing different T-2/BSA ratio and the use of alternate methods for the preparation of conjugates were considered. Therefore, T-2 HC-BSA and T-2 HS-BSA conjugates with different molar ratios of T-2 HS to BSA were prepared in the third quarter. Those conjugates have been delivered to the contracting officer and also have been used in our laboratory for antibody production.

The second important factor regarding the sensitivity and effectiveness of ELISA is the mycotoxin-enzyme conjugate. Again, an adequate molar ratio is essential. In a preliminary study, we found that it is necessary to use a large amount of free VT for the displacement of IgG-VT-HRP complex. This problem might be due to the presence of too much VT in the peroxidase. Since a large amount of toxin-enzyme conjugate (required at least 1 mg of T-2-BSA for each analysis) was necessary to estimate the amount of trichothecene mycotoxin coupled to the enzyme, we presently feel that it is not feasible to analyze the actual amount of toxin coupled to enzyme. Nevertheless, we plan to optimalize the coupling conditions through variations in the toxin to enzyme ratio and reaction times. Different toxin-enzyme preparations will then be tested for their effectiveness in the ELISA.

The indirect ELISA was found to be a sensitive method for monitoring antibody titer. However, a problem associated with this technique is that sometimes free mycotoxin can not displace the bound IgG from the solid phase. This problem might be due to the quality of the IgG as well as the amount of toxin conjugated to the polylysine or hemocynin. To overcome this problem, we plan to purify the IgG by affinity chromatography as well as to study the correlation between the conjugates with different toxin to polylysine molar ratio and the efficiency of displacement by free toxin.

Table I. Immunochemical reagents delivered during first 9 months.

Reagent	Quantities	Properties	Method of Preparation
T2-HS-EDA-BSA	14 mg	19[a]	MA[b]
T2-HS-BSA	59.4 mg	19[a]	WSC[c]
T2-HS-BSA	24.1 mg	21[a]	MA
T2-HS-BSA	15.5 mg	10[a]	MA
T2-HS-BSA	30 mg	27[a]	MA
T2-HS-hemocyanin	20 mg	--[d]	MA
T2-HS-polylysine	4 mg	--[d]	MA
T2-HS-peroxidase	2.5 mg	--[d]	WSC
T2-HS-alkaline phosphatase	2.4 mg	--[d]	WSC
T2-antisera	34 ml	e	e
^{3}H-T2	1 mCi	14 Ci/m mole	e

(a) Moles of T2 per mole BSA.
(b) MA, mixed anhydride method.
(c) WSC, water soluble carbodiimide method.
(d) Molar ratio not determined, but shown to be bound with T2 antiserum.
(e) Antibody titer and methods of preparation were given in first quarterly report.

Table II. Thin layer chromatography properties of different new trichothecene.

Trichothecene	Solvent systems[a]	R_f value	Color of spot	Visualization
T-2 toxin	A	0.52	brown-black[b] blue fluorescence	sulfuric acid
T-2 HS	A	0.27[c]	brown	sulfuric acid
T-2 HG	A	0.27[c]	brown	sulfuric acid
DAS	B	0.51	yellow	sulfuric acid
DAS-HS	B	0.26[c]	yellow-brown	sulfuric acid
VT	C	0.72	d	d
O-CM-VT	C	0.35	e	e
8-OH-VT	C	0.61	f	f

(a) The following systems were used: (A) ethyl acetate:toluene, 3:1 (v/v); (B) chloroform:methanol, 95:5 (v/v); (C) ethyl acetate:methanol:acetic acid, 8:2:0.2 (v/v/v).

(b) After spraying with sulfuric acid and heating at 120°C, the color of the T-2 spot was brown-black. If the plate (silica gel 60 F_{254}) was put under 365 nm UV light, the spot would appear fluorescent blue.

(c) T-2 HS, T-2 HG and DAS-HS exhibited an extensive tailing. The R_f value of these derivatives was sensitive to acid present in the sample and also the solvent system. The R_f values reported here were estimated from the middle of the tailing spot.

(d) Under 254 nm UV light, vomitoxin gave a black spot on a silica gel 60 F_{254} plate without any spraying. After sprayed with sulfuric acid and heated at 120°C, the spot turned a brown-yellow. When the plate was sprayed with 1% 4-(p-nitrobenzyl) pyridine in $CHCl_3$:CCl_4 (2:3), dried, heated at 150°C for 30 min, cooled, and sprayed with tetraethylenepentamine in $CHCl_3$:CCl_4 (2:3), VT and other related epoxytrichothene appeared as a blue spot.

(e) Same as (c) except the color of O-CM-VT was brown when sprayed with sulfuric acid.

(f) 8-OH-VT appeared as a blue spot when 4-(p-nitrobenzyl) pyridine and tetraethylenepentamine were used. A brown spot was observed after charring with sulfuric acid.

Table III. R_f values of saxitoxin derivatives in different TLC system[a].

Systems	STX	STXOL	STXOL-HS	STXOL-HS (Methyl ester)
1	0.48	0.56	0.68	--
2	0.23	--	0.80	0.67
3	0.55	0.32	0.7?	--
4	0.33	0.29	0.79	0.65
5	0.32	0.25	--	--

(a) TLC systems used are listed as follows:

1. C_{18} reversed phase plate; $CH_3OH:HOA_c:H_2O$ (10:2.5:4).
2. C_{18} reversed phase plate; CH_3OH:5% HOA_c (5:4).
3. Silica gel G60 F254 plate; $ETOH:H_2O:HOA_c$ (5:5:1.5).
4. Silica gel G60 F254 plate; $Acetone:HOA_c:H_2O$ (10:3:3).
5. Silica gel G60; $Butanol:HOA_c:H_2O$ (2:1:1).

Table IV. Production of antibodies against VT, DAS, T-2 toxin and STX.

Conjugates used	No. of rabbits tested	Weeks of immunization	No. of boosters	Titers[a]		
				RIA	Direct ELISA	Indirect ELISA
T2-HS-EDA-BSA	2	27	--	Y	Y	Y
T2-HS-BSA	3	6	1	Y	Y	NT
T2-HG-BSA	3	1	0	NT	NT	NT
O-CM-VT-BSA (MA)[b]	6	20	3	N	N (YW)	Y
O-CM-VT-EDA-BSA (WSC)[c]	3	11	2	NT	N	N
O-CM-VT-BSA (WSC)	3	11	2	NT	Y	Y
STX-HS-BSA (MA)	3	11	2	N	Y	Y
DAS-HS-BSA (MA)	3	13	2	NT	Y	Y

(a) Abbreviation used: Y, yes; NT, not determined; YW, yes, but weak; N, no.
(b) MA, prepared by mixed anhydride methods.
(c) WSC, prepared by water soluble carbodiimide method.

Table V. Toxicity of T-2 toxin and hemisuccinate of T-2 toxin[a].

Dose mg/kg	No. of animals dead in two days	
	T-2 toxin	T-2 HS
1	0	0
2.5	1 (25)[b]	0
5	4 (100)	1 (25)
7.5	--	2 (50)
10	--	4 (100)

(a) Four male mice with body weight of 25-30 g per group were each injected with various amounts of toxin in 0.1 ml of DMSO.
(b) Values in parentheses indicate % of total.

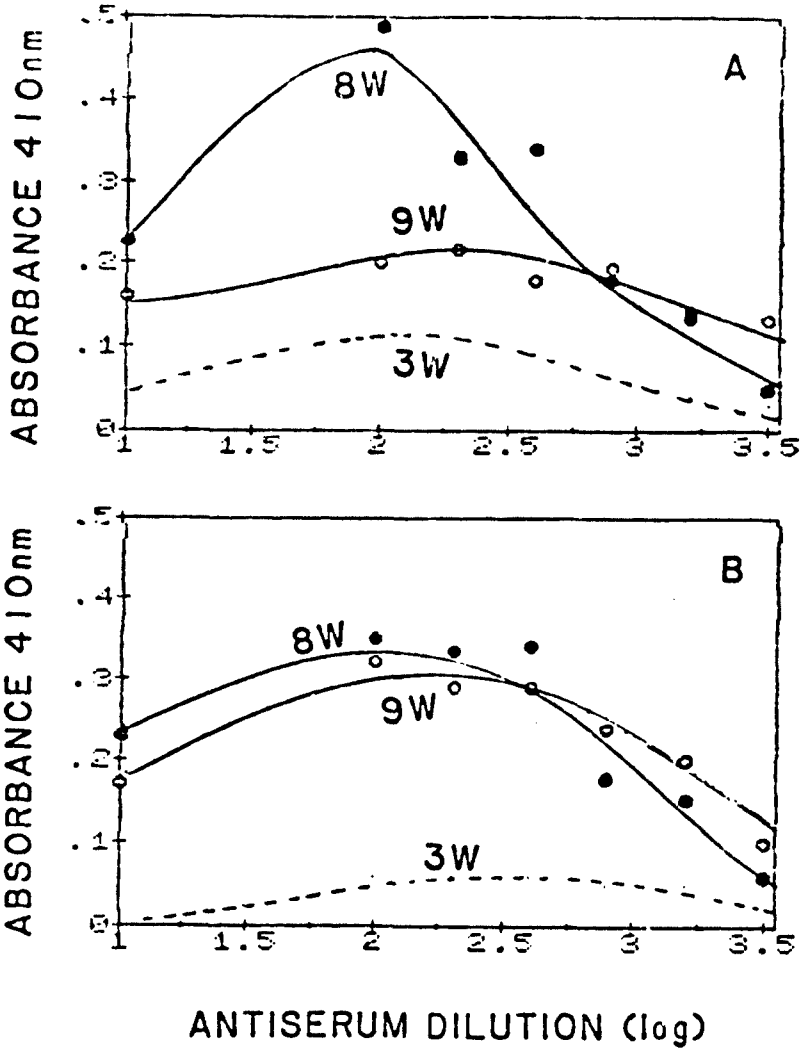

Figure 1. Determination of antibody titers by direct ELISA. Figs. 1A and 1B are the antibody titers for rabbits TZ-1 and TZ-2 after immunizing with O-CM-VT-BSA (prepared by WSC method) for 3, 8 and 9 weeks. A booster injection was made on the 7th week. The assay was carried under the conditions similar to those for aflatoxin B1 except that the antisera of different dilution was coated to an Immulon plate (Dynatech) by the bicarbonate method. O-CM-VT-HRP (0.5 μg/well) was used as an enzyme marker.

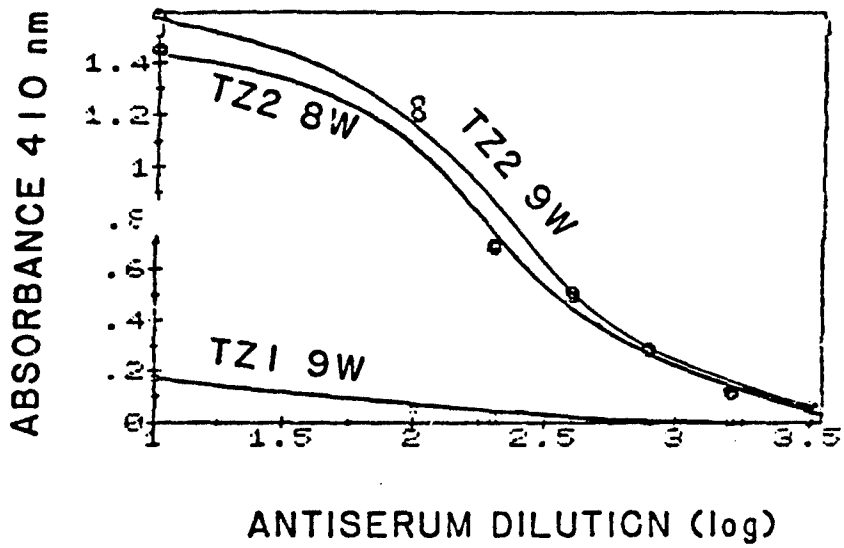

Figure 2. Determination of antibody titers by indirect ELISA. Antisera obtained from rabbit TZ-1 9 weeks (lower curve), or rabbit TZ-2 8 (middle curve) or 9 weeks (upper curve) after immunization were used in the analysis. O-CM-VT-polylysine (50 µl of 1 to 500 dilution; or 0.2 µg/well) was coated to Immunlon I (Dynatech) by the bicarbonate method. Antisera of different dilutions in phosphate buffer were then incubated with the coated plate at 37C for 1 hour, followed by washing with buffer, and reincubated with 50 µl of goat antirabbit IgG-HRP (1 to 500 dilution of Sigma preparation cat. no. A8275) for another hour at 37C. After washing with phosphate buffer, 100 µl of ABTS substrate was added and incubated at 37C for another hour. The reaction was stopped by the addition of 100 µl of HF-EDETIC acid and absorption at 410 nm determined.

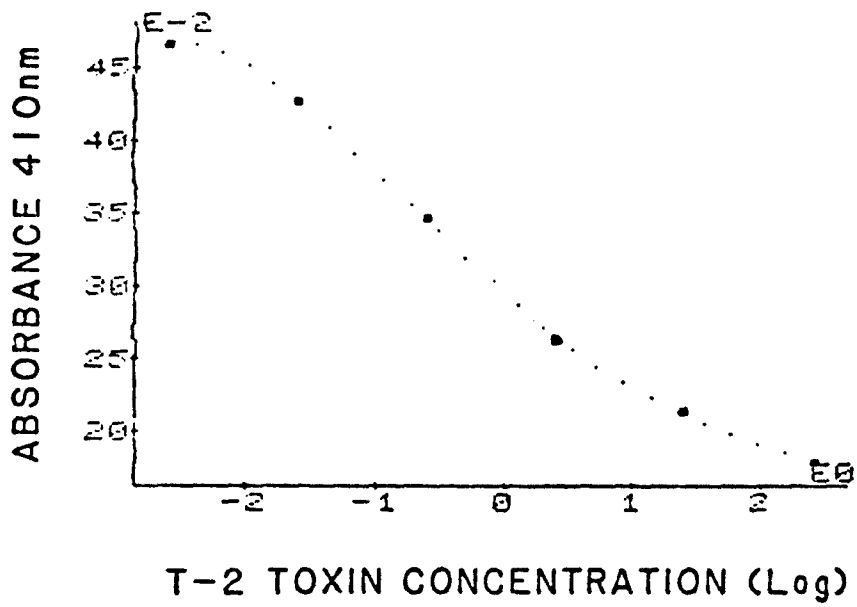

Figure 3. Indirect ELISA of T-2 toxin. The analysis was carried according to the protocol described in Figure 2 except that T-2 HS-polylysine (0.3 µg/well) was coated to the plate and that T-2 antibody at a dilution of 1 to 5,000 (50 µl/well) was incubated together with 50 µl of various T-2 toxin in the second step. The minimal detection level for T-2 toxin is around 12.5 pg in each assay (or 0.5 ng/ml).